



BREAKING THE SILENCE ABOUT THE BIOFILM

*Raghavendra Rao M.V¹ | Mohammed Khaleel³ | Manik Dass S⁵ | Khizer Hussain Junaidy⁴ | Amreen⁶ | Mahendra Kumar Verma⁷ | Dilip Mathai²

¹ Scientist-Emeritus and Director, Central Research Laboratory, Apollo Institute of Medical Sciences and Research, Jubilee Hills, Hyderabad, Telangana, India. (*Corresponding Author)

² Professor, Department of Medicine and Dean, Apollo Institute of Medical Sciences and Research, Jubilee Hills, Hyderabad, Telangana, India.

³ Professor of Microbiology, Clinical & Diagnostic Microbiologist, Department of Microbiology, Deccan College of Medical Sciences, Hyderabad, TS, India

⁴ Resident, Department of Pharmacology, Gandhi Medical College, Hyderabad, TS, India.

⁵ Professor and Head, Department of Microbiology, Apollo Institute of Medical Sciences and Research, Jubilee Hills, Hyderabad, Telangana, India.

⁶ Resident, Department of Paediatrics, Osmania Medical College/Niloufer Hospital, Hyderabad, India.

⁷ Department of Biotechnology, Acharya Nagarjuna University, Guntur, AP, India.

ABSTRACT

Microorganism evolved with syntrophic consortium called as bio film which provides an beneficial environment for their growth. The biofilm formation is often considered as an adaptation of large number of microbes against unfavourable environmental conditions. However, there is prevailing scientific evidence suggesting role of biofilm in offering transport of essential molecules including nutrients. Research findings have demonstrated that microbial cells are capable of producing variety of substances which serve as building blocks, for biofilm synthesis and cells get embedded into the matrix of extracellular polymeric substances (EPS) (1,2). These multicellular microbial arrangements allow microbes to communicate within the local environment as well as the external environment.

Certainly a biofilm promotes better growth and development of microbes. Also such arrangement provides microbes a protection against antibiotics and other antimicrobial agents. (3) Microbes are capable to adhere with all microbial adherence to biotic material a crucial step in microbial colonization is improved with biofilms, subsequently improving their growth. (4) The research findings have demonstrated that microbes capable of Biofilm formation attach to various living tissues including external and visceral. The adherence allows host microbes interaction and pathogenicity as well. The capacity of microbial pathogenicity is function of expression of virulence factors and these virulence factors are directly linked with quorum sensing and biofilm formation (5).

KEYWORDS: Planktonic, Cystic fibrosis (CF) *Pseudomonas aeruginosa*, *Helicobacter pylori*, Quorum-sensing systems, c-di-GMP, "Antimicrobial agent".

Introduction:

Biofilms have been intensively studied as communities of bacteria able to survive in environments unfavorable to planktonic (free-living) bacteria. (6)

Biofilms are congregation of heterogenous population of microorganisms that are surface associated and encapsulated in a self produced matrix. (7)

Biofilm related infections are emerging due to low susceptibility of microbial consortia to anti microbial drugs and host immune responses. (8)

Biofilm forming bacteria play a vital role in causing infections diseases and for enhancing the efficiency of the bioremediation process through immobilization. (9)

Bacteria form biofilm primarily during infectious diseases and in effluent treatment plants. It helps to retain a large population, which can withstand harsh environmental stress conditions. (10)

Bacteria within the biofilm can tolerate up to 1000 times more antibiotic concentrations compared to their planktonic partners. (11)

During effluent treatment process, a biofilm enables the bacteria to tolerate high concentrations of salts and organic compounds. Biofilm formation can be either regulated by the phenomenon of quorum sensing (High cell density) or it may may be independent of it. There a few methods and specific media, which allow bacteria to form a biofilm. Brain heart infusion and tryptic soy broth etc. (12)

For instance, *Pseudomonas aeruginosa* is the predominant cause of chronic airway infection in cystic fibrosis (CF). CF airway isolates are often tested for antibiotic susceptibility but are rarely eradicated by the antibiotics identified as potentially effective. The growth state of *P. aeruginosa* in CF airways is probably different from that exhibited under conventional susceptibility testing conditions and may represent a bacterial biofilm. Biofilm susceptibility testing methods were adapted to create an assay for implementation in a clinical microbiology lab-

oratory. (13)

Scientists isolated a novel aerobic chemolithotrophic sulfur-oxidizing bacterium, designated strain SO07, from wastewater biofilms growing under microaerophilic conditions. For isolation, the use of elemental sulfur (S(0)), which is the most abundant sulfur pool in the wastewater biofilms, as the electron donor was an effective measure to establish an enrichment culture of strain SO07 and further isolation. (14)

Bacteria growing in biofilms often develop multicellular, three-dimensional structures known as microcolonies. Complex differentiation within biofilms of *Pseudomonas aeruginosa* occurs, leading to the creation of voids inside microcolonies and to the dispersal of cells from within these voids. However, key developmental processes regulating these events are poorly understood. (15)

Current research suggests that the environmental signals regulating whether bacterial cells will initiate a biofilm differ from one bacterial species to another. This may allow each bacterial species to colonize its preferred environment efficiently (16)

Bacterial biofilms are communities of microorganisms attached to a surface. Biofilm formation is critical not only for environmental survival but also for successful infection. *Helicobacter pylori* is one of the most common causes of bacterial infection in humans. Some studies demonstrated that this microorganism has biofilm forming ability in the environment and on human gastric mucosa epithelium as well as on *in vitro* abiotic surfaces. (17)

Recently, some studies have alluded to the ability of *H. pylori* to form biofilms *in vitro*. In addition, *H. pylori* can form biofilms on the human gastric mucosa. Moreover, *H. pylori* could be embedded in drinking water biofilms on the surfaces of water distribution systems in developed and developing countries. (18)

In *Pseudomonas aeruginosa* outer membrane vesicle, (OMVs) have multifunctional biological roles including microbial interaction host infection

as well as maintenance of the structure of biofilm. (19)

Pseudomonas aeruginosa is considered to grow in a biofilm in cystic fibrosis (CF) chronic lung infections. Bacterial cell motility is one of the main factors that have been connected with *P. aeruginosa* adherence to both biotic and abiotic surfaces (20)

Type IV pili are expressed by a wide range of prokaryotes, including the opportunistic pathogen *Pseudomonas aeruginosa*. These flexible fibres mediate twitching motility, biofilm maturation, surface adhesion, and virulence. The pilus is composed mainly of major pilin subunits while the low abundance minor pilins FimU-PilVWXE and the putative adhesin PilY1 prime pilus assembly and are proposed to form the pilus tip. (21)

Microbial biofilms are mainly implicated in etiopathogenesis of caries and periodontal disease. Owing to its properties, these pose great challenges. Continuous and regular disruption of these biofilms is imperative for prevention and management of oral diseases. (22)

Biofilm research has gained immense importance in recent times due to the instrumental effects on health and disease manifestations. Several microbial infections have been associated with biofilm formation and pose challenges in treatment regimes (23,24)

Strategies to induce biofilm dispersal are of interest due to their potential to prevent biofilm formation and biofilm-related infections. Nitric oxide (NO), an important messenger molecule in biological systems, was previously identified as a signal for dispersal in biofilms of the model organism *Pseudomonas aeruginosa*. (25)

First small steps of history-- research on microbial biofilms:

Biofilms were actually observed long ago, before anyone had a clue what they were or how important they were. Indeed, at the time, no one really knew what science was, let alone biofilms. One of the early scientists to study microscopic life was the Dutchman, Antonie van Leeuwenhoek. He was well known for his ability to build microscopes that were quite a bit better than the run of the mill, the construction of which he held as a closely guarded secret. After utilizing one of his own microscopes in 1684 he remarked in a report to the Royal Society of London on the vast accumulation of microorganisms he was able to see in dental plaque. The history of biofilm observed from the time of van Leeuwenhoek's discovery of bacteria in 1676 to the present. (26)

Since the pioneering investigations of Robert Koch (1843 - 1910) microbiologists have been investigating bacteria almost exclusively in single species planktonic culture.

"It is quite evident that for the most part the water bacteria are not free floating organisms, but grow on submerged surfaces; they are of the benthos rather than the plancton." (Henrici 1933). And in a 1940 issue of the Journal of Bacteriology, authors H. Heukelekian and A. Heller wrote, *"Surfaces enable bacteria to develop in substrates otherwise too dilute for growth.*

This bacterial adhesion was accompanied by a decrease in the amount of suspended organic material. ZoBell concluded that the bacteria attached themselves to the glass surface where they encountered a concentration of organic nutrients much higher than would have been found in the bulk fluid (ZoBell 1943).

The observation of aggregated microbes surrounded by a self-produced matrix adhering to surfaces or located in tissues or secretions is old since both Leeuwenhoek and Pasteur have described the phenomenon. In environmental and technical microbiology, biofilms, 80-90 years ago, were already shown to be important for biofouling on submerged surfaces, for example, ships.

The concept of biofilm infections and their importance in medicine was, however, initiated in the early 1970s by the observation of heaps of *Pseudomonas aeruginosa* cells in sputum and lung tissue from chronically infected cystic fibrosis patients. The term 'Biofilm' was coined by Bill Costerton in 1978.

The term biofilm was introduced into medicine in 1985 by J. W. Costerton. During the following decades, the number of published biofilm articles and methods for studying biofilms increased rapidly and it was shown that adhering and non adhering biofilm infections are widespread in medicine. (27)

Condominium of Bacterial Biofilm:

The environment provided by biofilm is highly dynamic and flexible as per microbial need. It allows transport of essential elements including growth molecules i.e. nutrition and restrict entry of several invading pathogens and drugs i.e. antimicrobials and antibiotics.

The biofilm also provides an ideal environment for transport to nutrients to microbes in communication within the biofilm. The architecture of a biofilm is such unique that it allows each microbe inside to communicate with the neighbour. Based on habitat, microbe produces a variety of compounds which integrate into the biofilm. (28).

These molecules also differ in microbial species such as gram negative and gram positive. In a general finding it was told that gram negative bacteria are more effective in biofilm formation and hence more virulent compared to gram positive bacteria. (29).

Quorum-sensing systems and biofilm:

Quorum-sensing system an adopted communication system in microbes, that triggers biofilm formation. Quorum sensing (QS) signaling systems are responsible for the coordination of gene expression at a bacterial community level, which includes controlling the expression of virulence factors, as well as influencing the formation of biofilm. There is growing emphasis in developing anti quorum sensing molecules (inhibitors) instead of antibiotic to control microbial infections (30)

Research findings have demonstrated that microbial communities to form Biofilms makes them susceptible for antimicrobial agents and antibiotics. It is mainly due to higher exposure of antibiotics and antimicrobial agents to growing microbial populations. The findings also suggested in the absence of quorum sensing and biofilm formation microbes fail to express various virulence genes and protective gene/s that results in loss of microbial population (31).

Biofilm and pathogenicity:

For most of species of microbes including fungi and bacteria the virulence gene/s expression play crucial role in pathogenicity. The expression of virulence gene/s is function of stimulatory signal from external environment, internal environment or both. The housekeeping gene/s to a microbe sense a need for gene expression and external stimuli provide a direction selective gene expression (32)

Host tissue provide an ideal environment to microbes that colonize where they sense the need for gene expression.

For an example, *virbio cholera* a causative agent for Asiatic diarrhea has a complete set of virulence genes called TOXR. The expression of virulence gene/s in *Vibrio* are host dependent. This clearly signifies bacterial adaptation of human gut and surrounding environment. Studies shows same *Vibrio* in external environment do not express many genes of TOXR (33)

Biofilm and drug resistance:

Drug resistance is a growing challenge for mankind and posed a new threat to modern healthcare especially in the management of infections and infectious diseases. There are several factors associated with microbial drug resistance and bacterial adaptation such as biofilm formation is crucial one. Bacteria developed biofilm as one of the most important advantages in finding resistance against antimicrobials and antibiotic agents. It was reported that microbes capable in biofilm formation develop drug resistance to several hundred folds than non biofilm forming microbes (34,35).

Biofilm diffusion of antibiotic through the matrix. It was also reported that a large number of clinically approved antimicrobial agents are unable to enter the biofilm surface or move inside the environment. Here, larger concentrations of such agents are required to penetrate into deeper part of biofilm and provide bactericidal effect. viz, The antibiotics such as amino glycosides enter more slowly through the matrix of biofilm than β -lactams. Quorum sensing is an evolved mechanism of cross talk among microbes and works more efficiently in the biofilm. Hence it provides an ideal environment to microbes residing inside biofilm for the transmission of gene/s responsible for drug resistance. Many genetic mobile elements such as plasmids, transposons, can easily be transferred among microbes, inside the biofilm and well communicated via quorum sensing. Biofilm not only restricts entry of antimicrobials and antibiotics but also enables several mechanisms to pump out such antimicrobial agents from the matrix and provides an ideal growth environment (36,37)

Many antibiotics and antimicrobial agents require a cofactor for their activation. Though various metal ions & chemicals are rich in the Biofilm, they alter the Biofilm pH and disabling the antimicrobial agent. Further studies also show that drastic change in pH and concentration of metal ions antagonizes antimicrobial activity. Microbial biofilms are also rich in metabolically inactive cells called as persister cells. Cell that developed resistance to various harsh environmental stress. These cells remain in periphery of biofilm and actually neutralize effect of antimicrobial agents and offer a protection to cells present in the core. Further, studied such cells are highly tolerant to antibiotics forming a reservoir of surviving cells able to rebuild the biofilm population (38)

There are several other mechanisms in biofilm residing microbial cell against antimicrobial agents. The biofilm acts as reservoir for food and other nutrients. During anti-microbial impact, cells undergo slow metabolic events viz gene expression and protein synthesis. However, these cells remain viable for longer time as an ideal environment is provided by the biofilm (39)

The inactive microbial cells in the biofilm synthesize several toxins that often antagonize antimicrobial agents.

This is unique feature of microbes and biofilm acts as the crucial interface for

using such metabolically inactive cells in defence and survival.. There are several proteins needed to ensure existence of cells. These proteins are ideal targets for the discovery of novel molecules capable of effectively treating chronic and biofilm-related infections. The level of drug resistance also depends on development of biofilm. Hence targeting biofilm in initial stage will be more effective and easy in controlling microbial growth.. It allows exploring various building molecules of biofilm and targeting such molecules actually disable microbes from host integration. This also prevents microbes from down regulating the host immune system (40).

On the contrary, if microbe's succeeds in secretion of EPS and the attachment becomes irreversible, biofilm is more resistant to antibiotics and host immune responses. The biofilm existence is an advantage to pathogen in finding an escape from host immune surveillance and impact of antibiotics. The architecture of microbial biofilm is unique and evolved to nourish entire microbial colonies by water channels that help to distribute nutrients and signaling molecules (41).

Architecture of Biofilm:

As stated above the biofilm formation is complex and requires five steps to complete it. There are numerous factors and player involved in biofilm formation and most of them are microbial origin however host factors are also crucial here (42,43).

The first attachment of bacteria is influenced by attractive or repelling forces that vary depending on nutrient levels, pH, and the temperature of site or niche. In this step, flagella and chemotaxis play an important role avoiding the action of the hydrodynamic and repulsive forces as well as selecting the surface, respectively. Irreversible attachment to surfaces. In the case of *E. coli*, it is mediated by type 1 pili, curli fibres, and antigen 43 that also favours the interbacterial interactions. In the case of *P. aeruginosa* as well as other *Pseudomonas* species, transition from reversible to irreversible attachment has been well studied. It has been observed that *P. fluorescens* requires an ATP-binding cassette (ABC) encoded by the *lap* genes for carrying out this process (44)

EPS, adhesins, amyloid-forming proteins, and exo polysaccharides (all included in biofilm matrix) are required to generate these structures in which gradients of nutrients, water, signalling compounds or waste products are present along the different areas of biofilm, conditioning the metabolism of the cells. When biofilm is fully mature, detachment may occur. Detachment allows cells to again take on a planktonic state and can thereby form biofilm in other settings. It has been proposed that bacteria detachment could be caused by active mechanisms initiated by the bacteria themselves such as enzymatic degradation of the biofilm matrix and quorum sensing in response to environmental changes related to nutrition levels and oxygen depletion and by passive mechanisms mediated by external forces and erosion (45)

Biofilm dispersal is an important step in a high number of bacterial species, allowing their transmission from environment to human host, between hosts, and even within a single host spreading the infection. The role of c-di-GMP levels in the biofilm dispersion has recently been determined, being a second messenger used in signal transduction in a high number of bacteria species. Thus, it has been proposed that high levels of c-di-GMP increase the sessile behavior of the bacteria, while low levels increase the motility of the bacteria. c-di-GMP affects EPS production, biofilm formation, cell length, and swimming motility in *E. Coli*. (46).

Current research:

The current focus is on finding anti biofilm drug development. It also includes developing novel molecules that restrict secretion of various biofilm forming elements. The most challenging aspect of current infection diseases is treatment of urinary tract infections and associated diseases. UTI is caused by various gram positive and gram negative microbes' primarily bacteria. However, most of these causative agents result in successful colonization in lower and upper urinary tract via biofilm formation in vital tissue (47,48).

Current research emphasis is finding molecules that can target biofilm formation.. In this regard, various polysaccharides are coupled with antimicrobial agents and antibiotics to tackle one of most serious,yet frequent health complication to humans. . The role of quorum sensing in biofilm formation and involvement of other molecules is underway in modern research (49,50).

Biofilm formation is a complex process involving several mechanism and molecules in host tissue.

Research on agents that inhibits bacterial biofilm formation:

In the biofilm form, bacteria are more resistant to various antimicrobial treatments. Bacteria in a biofilm can also survive harsh conditions and withstand the host's immune system. Therefore, there is a need for new treatment options to treat biofilm-associated infections. Currently, research is focused on the development of anti biofilm agents that are nontoxic, as it is believed that such molecules will not lead to future drug resistance. In this review, we discuss recent discoveries of antibiofilm agents and different approaches to inhibit/disperse biofilms. These new antibiofilm agents, which contain moieties such as

imidazole, phenols, indole, triazole, sulfide, furanone, bromopyrrole, peptides, etc. have the potential to disperse bacterial biofilms in vivo and could positively impact human medicine in the future (51)

Biofilm-associated bacteria are less sensitive to antibiotics than free-living (planktonic) cells. Furthermore, with variations in the concentration of antibiotics throughout a biofilm, microbial cells are often exposed to levels below inhibitory concentrations and may develop resistance.

The Centers for Disease Control and Prevention use the terms "antibiotic" and "antimicrobial agent" interchangeably (52)

On the other hand, the misuse of antibiotics also contributed to development of drug resistance, which might aggravate the bacteria infected disease. Thus, novel strategies other than antibiotics should be developed to combat the bacterial and biofilm formation. In last two decades, novel approaches in preventing biofilm formation and QS have been widely developed and reported including natural products from plants. Many plant natural products have been demonstrated antimicrobial and chemo-preventive properties (53)

It is well known that herbal remedies have been employed by different human cultures for centuries and some of those natural products are essential for prevention and treatment of infectious diseases. For example, traditional Chinese medicinal herbs were commonly used in bacterial infection and prevention and some herbs viz as *Scutellaria*, *Taraxacum* and *Tussilago* exhibited antibacterial ability (54)

Recently, extracts from plants were also reported to regulate biofilm formation and inhibit QS (55)

New and future research developments in microbial biotechnology:

Microbial Cellulase System Properties and Applications covers the biochemistry of cellulase system, its mechanisms of action, and its industrial applications. Research has shed new light on the mechanisms of microbial cellulase production and has led to the development of technologies for production and applications of cellulose degrading enzymes. The biological aspects of processing of celullosic biomass have become the crux of future research involving cellulases and cellulolytic microorganisms, as they are being commercially produced by several industries globally and are widely being used in food, animal feed, fermentation, agriculture, pulp and paper, and textile applications (56)

Biofilm Emerging research in nanotechnologies in immunology:

Bacterial colonization in the form of biofilms on surfaces causes persistent infections and is an issue of considerable concern to healthcare providers. There is an urgent need for novel antimicrobial or antibiofilm surfaces and biomedical devices that provide protection against biofilm formation and planktonic pathogens, including antibiotic resistant strains. In this context, recent developments in the material science and engineering fields and steady progress in the nanotechnology field have created opportunities to design new biomaterials. (57)

The future aim on biofilm based research will be to understand nature of biofilm and its formation. More emphasis must be given on the nature of molecules and chemical involved in biofilm formation. The failure of a large number of clinically approved antibiotics and antimicrobial agents provided us a lesson to look into another aspect and new paradigm shift in studying microbial drug resistance. Classical example is urinary tract infection where variety of gram positive and gram negative microbes get colonize and form biofilm, leading to serious health issue. Using antibiotic and combination of antibiotics along with new generation drugs will not be sufficient to deal with "antimicrobial resistance" until the mechanism of drug resistance. Certainly biofilm formation is crucial for developing drug resistance in a large species of microbial world.(58)

Efforts are also on going to create Nanotechnology based biomaterial with anti-fouling ,bactericidal and anti-biofilm properties. These materials being resistant to biofilm formation and being bio-compatible,non-toxic and cost effective serve advantageous in making bio-medical devices (59).

Research strategies for the prevention of microbial biofilm formation:

Total laryngectomy, a surgical treatment for extensive cancer of larynx, which alters swallowing and respiration in patients, is followed up with a surgical voice restoration procedure comprising tracheoesophageal puncture techniques with insertion of a "voice prosthesis" to improve successful voice rehabilitation. However, microbial colonization is a major drawback of these devices. Antimicrobials are usually used to prevent the colonization of silicone rubber voice prostheses by microorganisms. However, long-term medication induces the development of resistant strains with all associated risks and the development of alternative prophylactic and therapeutic agents, including pro biotics and bio surfactants, have been suggested. The inhibition of microbial growth on surfaces can also be achieved by several other techniques involving the modification of physicochemical properties of the biomaterial surface or the covalently binding of antimicrobial agents to the biomaterial surface.(60)

Future research perspective:

As we look into the future of microbial biofilm research, there is clearly an emerging focus on communities rather than populations. This represents an essential change in direction to more accurately understand how and why micro-organisms assemble into communities, as well as the functional implications for such a life style. For example, current research studies shows that communities display emergent properties or functions that are not predicted from the individual single species populations, including elevated stress tolerance and resistance to antibiotics. Models for mixed species biofilms can be very simple, comprised only a handful of species or can be extremely species rich, with hundreds or thousands of species present. The future holds much promise for this area of research, where investigators will increasingly be able to resolve, at the molecular and biochemical levels, interspecies relationships and mechanisms of interaction. The outcome of these studies will greatly enhance our understanding of the ecological and evolutionary factors that drive community function in natural and engineered systems. (61)

Shortened version of a large work:

Biofilm associated microbial infections are subject of major concern in health, food, agriculture and industrial sectors .Attributes like biofilm dispersal and spread of infection, high efficiency of genetic exchange via Environmental DNA,(eDNA), reduced susceptibility to anti microbial agents, enhanced ability to form endotoxins(in case of gram negative bacteria) and inherent resistance against host immune system make inhibition of biofilm infections a tough ordeal (62)

Natural detergents with Biofilm encoding properties could be developed by cleaning Biofilms through sanitization in health, food and dairy industries. Last two decades of path breaking research in this field has uncovered various aspects of this complex social life style of bacteria

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